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# Cytological Basis of Photoresponsive Behavior in a Sponge Larva

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**Abstract.** Ontogenetic changes in the photoresponse of larvae from the demosponge *Reneira* sp. were studied by analyzing the swimming paths of individual larvae exposed to diffuse white light. Larvae swam upward upon release from the adult, but were negatively phototactic until at least 12 hours after release. The larval photoreceptors are presumed to be a posterior ring of columnar monociliated epithelial cells that possess 120-$\mu$m-long cilia and pigment-filled protrusions. A sudden increase in light intensity caused these cilia to become rigidly straight. If the light intensity remained high, the cilia gradually bent over the pigmented vesicles in the adjacent cytoplasm, and thus covered one entire pole of the larva. The response was reversed upon a sudden decrease in light intensity. The ciliated cells were sensitive to changes in light intensity in larvae of all ages. This response is similar to the shadow response in tunicate larvae or the shading of the photoreceptor in *Euglena* and is postulated to allow the larvae to steer away from brighter light to darker areas, such as under coral rubble—the preferred site of the adult sponge on the reef flat. In the absence of a coordinating system in cellular sponges, the spatial organization and autonomous behavior of the pigmented posterior cells control the rapid responses to light shown by these larvae.

## Introduction

Light, gravity, current, and chemical cues enable the larvae of many marine invertebrates to locate the habitat that will best ensure their success as adults (Grave, 1926; Ryland, 1960; Thorson, 1964; Forward and Costlow, 1974; Brewer, 1976; Young and Chia, 1982; Miller and Hadfield, 1986; Svane and Young, 1989; Pawlik, 1992). Thus, eyespots are well developed in many bilaterian larvae (see Eakin, 1968, 1972; Burr, 1984), and signals received by these and other sensory organs are apparently translated into behavior *via* the larval nervous system (Thomas *et al.*, 1987; Kempf *et al.*, 1997; Murphy and Hadfield, 1997; Hadfield *et al.*, 2000). The role of photosensory systems in the larval behavior of basal metazoans is less well documented. Although ocelli are well developed in cnidarian medusae and polyps (Thomas and Edwards, 1991), the putative photoreceptors that have been identified in planulae are simple monociliated sensory cells with electron-dense granules (Weis *et al.*, 1985; Thomas *et al.*, 1987). Presumably the neurons underlying the ciliated epithelium of cnidarian planulae are involved in assessing the environment (Chia and Koss, 1979; Martin and Chia, 1982; Thomas *et al.*, 1987), but there is currently no evidence for synaptic signaling between presumptive photoreceptors and other cells. Poriferan larvae are considered to be even more simply constructed than planulae in that they lack neurons.

Porifera is the only metazoan phylum that lacks neurons (Pavans de Ceccatty, 1974a, b; Mackie, 1979). Furthermore, despite one report suggesting electrical coupling between two reaggregated cells from dissociated adult tissue (Loewenstein, 1967), there is no evidence that sponges have gap junctions, which would allow the rapid conduction of behavioral signals between cells (Green and Bergquist, 1982; Lethias *et al.*, 1983). Members of the subphylum Symplasma, the Hexactinellida, are the only sponges known to be capable of rapid behavior (Lawn *et al.*, 1981; Mackie *et al.*, 1983). Because hexactinellid tissue is mostly syncytial (Leys, 1995), the electrical signals that cause concurrent shutdown of flagellar activity propagate along the membrane of the continuum (Leys and Mackie, 1997; Leys *et al.*, 1999).

Behavior in cellular sponges, the Demospongiae and Cal-

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carea, is limited to gradual contraction of the tissues (McNair, 1923; Vacelet, 1966; Pavans de Ceccatty, 1969, 1976; Mackie, 1979; Lawn, 1982) and variations in pumping patterns (Reiswig, 1971), for which chemical or mechanical coordination are invoked. Although the mechanisms for coordinated behaviors are apparently absent, cellular sponge larvae do exhibit rapid responses to external stimuli. The responses of sponge larvae to light, gravity, and current have been reported since the early 1900s (reviewed in Wapstra and van Soest, 1987).

Photokinesis, one of the most tangible aspects of sponge larval behavior, is best known from studies on parenchymellae larvae of demosponges (Warburton, 1966; Bergquist and Sinclair, 1968; Bergquist *et al.*, 1970; Wapstra and van Soest, 1987; Woollacott, 1990, 1993; Maldonado and Young, 1996, 1999). Typically, these larvae are oblong and heavily ciliated. The parenchymellae of different species are distinguished primarily by the presence or absence of cilia at the poles of the larva, of a ring of longer cilia at one end, or of pigmented cells at one end. Unfortunately, the pattern of ciliation or pigmentation on larvae smaller than 500 $\mu$m is difficult to determine accurately by light microscopy, and relatively few larvae have been characterized by electron microscopy (Evans, 1977; Simpson, 1984; Woollacott and Hadfield, 1989; Harrison and De Vos, 1991; Kaye and Reiswig, 1991; Amano and Hori, 1992; Woollacott, 1990, 1993; Fell, 1997). Furthermore, only a few investigators have taken an experimental approach to sponge larval behavior (Jaeckle, 1995; Woollacott and Hadfield, 1996; Maldonado and Young, 1996, 1999; Maldonado *et al.*, 1997); most studies report only anecdotal observations.

The cellular mechanisms underlying sponge larval behavior have yet to be addressed: how does an animal lacking nerves and communicating junctions between its cells respond so agilely to light and other stimuli? This paper addresses the ontogenetic change in the light response and its cytological basis in the parenchymella larva of the demosponge *Reneira* sp.

## Materials, Methods, and General Observations

### *Collection and maintenance of specimens*

Adult specimens of the sponge *Reneira* sp. (Porifera, Demospongiae, Haplosclerida, Chalinidae) were collected in February, April, August, and December, 1999, from the reef flat in Shark Bay on Heron Is. Reef, Great Barrier Reef (23°26′N, 151°03′E). The sponges were maintained in shaded aquaria in seawater pumped from the reef slope.

### *Systematics*

The identification of this sponge as *Reneira* sp. was confirmed by taxonomists at the Queensland Museum. However, as this species has not yet been formally described, a brief description is given here. The sponge is grey or olive brown, and its texture is firm due to a well-developed anisotropic reticulate network of primary spongin that is cored by paucispicule to multispicule tracts of oxeas 80–100 $\mu$m long by 1 $\mu$m wide. Oscula are slightly raised above the surface of the sponge, which is formed by a typical chalinid isodictyal reticular network that is tangential to the surface. We have deposited a voucher specimen and photograph in the Poriferan Collection in the Queensland Museum (QM G315611). The North Atlantic genus *Reneira* has been variously called *Haliclona* or *Adocia* in the past, and most recently taxonomists have formally transferred the genus *Reniera* to *Haliclona* (de Weerdt, 1986). Although the Pacific species of these genera have not been revised recently (J. Hooper, Queensland Museum, Australia; pers. comm.), the behavior and structure of the *Reneira* sp. larvae studied here appear to be very similar to those reported for other chalinids, and even most haplosclerids (Wapstra and van Soest, 1987).

### *Habitat and description of adult sponges*

The sponge forms encrustations 1–3 cm thick on the underside of coral rubble, which is home to numerous other encrusting and grazing animals. The coral is just submerged at low tide and is approximately 3 m deep at high tide.

The brood chambers of *Reneira* sp. are typically located in the lowest portion of the sponge closest to the coral substrate (Fig. 1a). *Reneira* sp. is reproductive year round (Leys and Degnan, unpubl. data), but although sponges collected in all seasons contained brood chambers, sponges collected in August had the least number and released the fewest larvae. The chambers are up to 1 $cm^2$ in diameter and contain 20 to 150 embryos, 600–900 $\mu$m long, in a wide range of developmental stages (Fig. 1b). Spermatocysts were found in only 2 of more than 100 sponges that were collected and sectioned during all collection periods.

### *Description of the larvae*

The larvae of *Reneira* sp. are cream colored with a dark ring of pigment-containing cells around the posterior end; in fact, the dark pigmented ring defines the posterior end (Fig. 1b, c). The outer layer of the larva consists primarily of monociliated cells possessing 20-$\mu$m-long cilia (hereafter called short lateral cilia), but there are two protruding bare patches, one each at the poles of the larva. The bare patch at the anterior end is 55–60 $\mu$m in diameter, and that at the posterior end is 140–160 $\mu$m in diameter and lies inside the pigmented ring (Fig. 1c, d). The anterior border of the pigmented ring is marked by a ring of cells that contain pigment vesicles but also give rise to 120–150-$\mu$m long cilia (hereafter called long posterior cilia) (Fig. 1c, d). These latter structures are more appropriately described as cilia

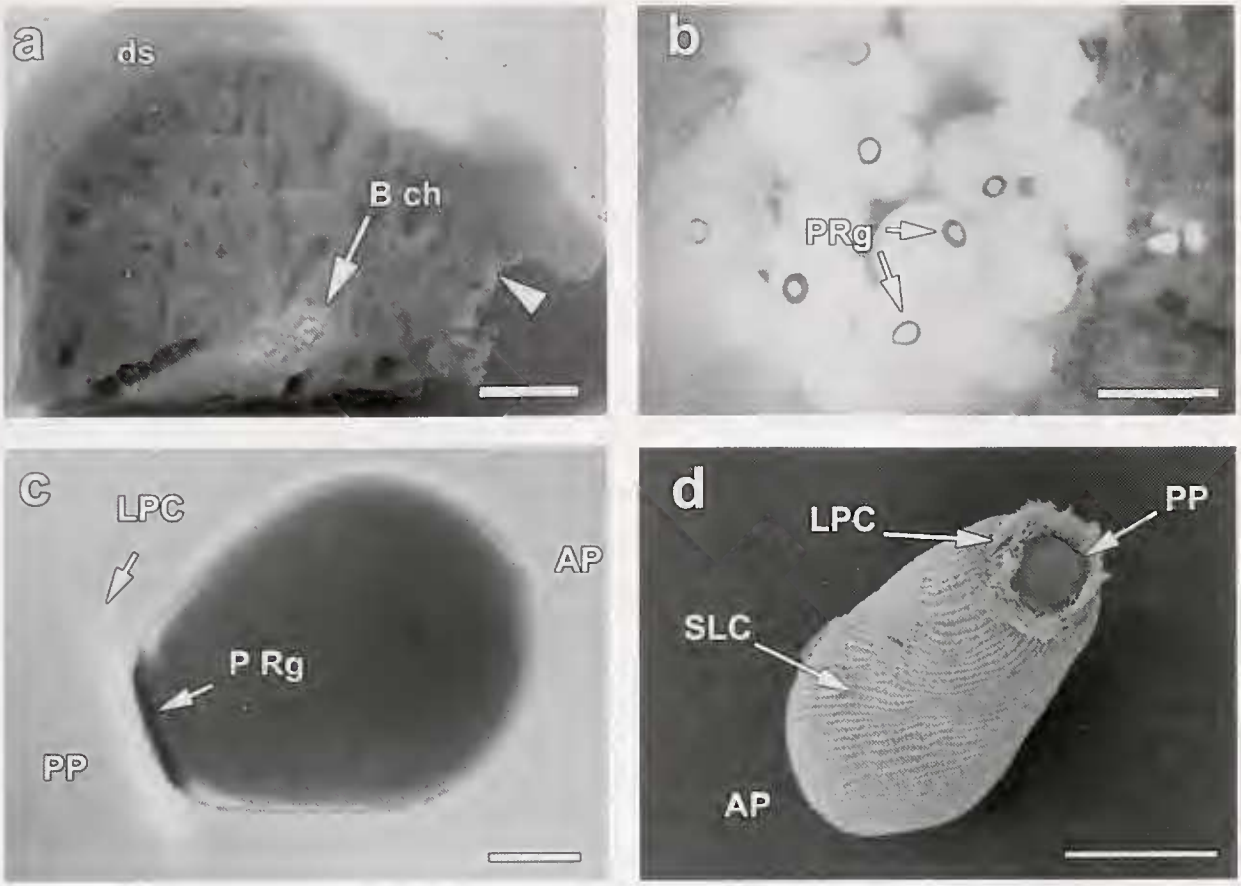


**Figure 1.** Brood chambers and embryos of *Reneira* sp. in various stages of development (a–c: light microscopy; d: scanning electron microscopy). (a) A section of the adult sponge that was attached at its lower edge to the coral substrate (arrowhead) shows a brood chamber (B ch) with embryos and larvae. Bar: 1 cm. Dermal surface, ds. (b) Embryos and larvae in a brood chamber clearly showing the pigment ring (PRg) at one pole. Bar: 1 mm. (c) A swimming larva showing the dark pigmented ring (PRg) and long posterior cilia (LPC) at the posterior swimming pole (PP), and a protrusion at the anterior swimming pole (AP). Bar: 100 μm. (d) A larva showing the long posterior cilia (LPC), the unciliated posterior pole (PP), and the lines of short lateral cilia (SLC) arrested by fixation during their beat in metachronal waves. Bar: 250 μm.

rather than flagella because their motion is whiplike; they do not propagate quasi-sinusoidal waves (Alberts *et al.*, 1989).

*Laboratory experiments on larval phototaxis*

The larvae were maintained individually in 2 ml of 0.2-μm-filtered seawater in 12-well multiwell dishes at room temperature (about 22 °C). At various times after release—*i.e.*, 0, 2, 4, 6, 12, 24, and 48 hours—individual larvae were pipetted into a rectangular aquarium (15 × 20 cm) containing 0.2-μm-filtered seawater (Fig. 2a). Pipetting was not observed to affect the swimming behavior of the larvae. The rectangular aquarium (the test chamber) was immersed in seawater in a second aquarium, which was blackened on all but one side to reduce reflected light (after Wendt and Woollacott, 1999). Light from a cold light source (Volpi Intralux 5000) was passed through a diffuser made of acrylic plastic into the inner test chamber, such that a gradient of light was created in the horizontal direction from the front to the back of the test chamber (950 $\mu M \cdot$ photons $\cdot$ $m^{-2} \cdot s^{-1}$ to $<1$ $\mu M \cdot$ photons $\cdot$ $m^{-2} \cdot s^{-1}$). The radiance at the side closest to the light was at the same level recorded at the edge of the underside of a coral boulder at low tide on the reef flat in bright sunlight during the day (Fig. 2b). Light measurements were made in the field and in experimental aquaria with a LI-COR underwater quantum sensor (LI-192SA, LI-COR Inc., Nebraska). Ambient light in the room where measurements were made was 1.8 $\mu M \cdot$ photons $\cdot$ $m^{-2} \cdot s^{-1}$. A glass plate was placed above the test chamber, and the changing position of the larva in the test chamber was recorded for one minute with a nonpermanent felt marker; these records were later transcribed onto paper. Between tests, the larvae were maintained away from direct light in their multiwell dish, at 22 °C.

The initial direction swum by each larva was recorded

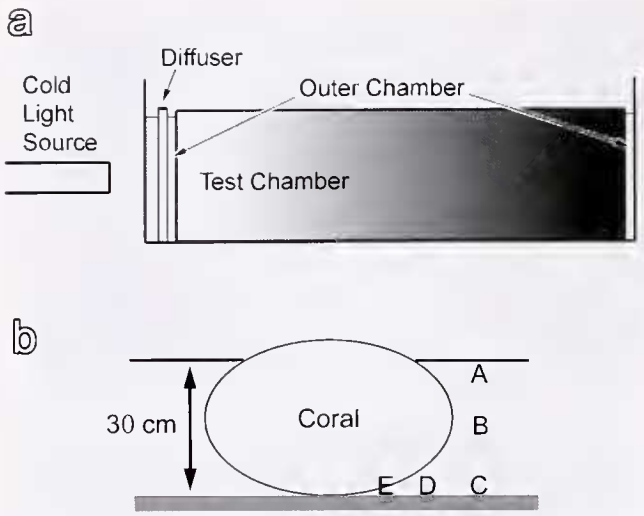


**Figure 2.** (a) The experimental apparatus for measuring the phototaxis of individual larvae in response to horizontal light from a cold light source shining through a diffuser of acrylic plastic. A test chamber containing filtered seawater is immersed in seawater contained in an outer chamber, which is blackened on all sides except that facing the light source. Larvae were dropped by pipette into the inner test chamber in which there was a gradient of light in the horizontal direction (left to right in the diagram) of 950 $\mu M \cdot$ photons $\cdot m^{-2} \cdot s^{-1}$ to $<1$ $\mu M \cdot$ photons $\cdot m^{-2} \cdot s^{-1}$. See methods for further details. (b) Light intensities on the reef flat during a sunny day were recorded at 5 positions (A–E) around coral rubble that was in 30 cm of water at low tide. The average of 10 measurements at each position is given in $\mu M \cdot$ photons $\cdot m^{-2} \cdot s^{-1}$. A: 1906.5; B: 1354; C: 785.8; D: 57.9; E: 9.4. The substrate below the coral was sand.

and plotted as a circular distribution. The mean angle swum by the larvae in each age group (*i.e.*, 0–48 h) was calculated, and the measure of randomness was tested using the nonparametric Rayleigh test [a high $z$ value, or an $r$ value approaching 1, indicates the data are highly grouped (Zar, 1984)].

*Video and light microscopy*

Live larvae were observed with an Olympus SZH dissecting microscope with a 1X plan lens, and with an Olympus BX60 compound microscope equipped with an Olympus C35 AD4 photoautomat. New glass coverslips (22 × 22 mm) were placed on the bottom of a 5-cm-diameter plastic petri dish, and individual larvae were pipetted forcefully onto this surface, causing them to adhere by their anterior end for up to 5 min. During this period, light levels could be manipulated, and the cilia could be observed. Cold light was shone on the posterior end of the larva. Other larvae that had adhered to the dish or coverslip were transected medially, creating an anterior portion and a posterior portion with its pigment ring and long posterior cilia intact. Although mucus and cellular material from the wound was initially caught in the cilia, these debris disappeared after several minutes; then cilia on both the anterior and posterior portions continued their normal beating, and both halves rotated as they did prior to being cut. If the posterior half of a bisected larva was placed with the pigment ring facing upward, it would continue to rotate on the spot indefinitely. Light from a cold light source was shone at the pigmented ring and long posterior cilia on the posterior end of the bisected larva from either the left or right side of the microscope stage. The ciliary beat was recorded using a Panasonic digital CCD video camera and a National time-lapse VCR (AG6010) in real-time recording mode. The intensity of light from the cold light source was measured in seawater on the dissecting microscope base with a LI-COR underwater quantum sensor.

The effect of elevated KCl (10–50 m$M$) on beating of cilia was tested; ciliary beating was recorded by video CCD.

*Electron microscopy*

Larvae were fixed for ultrastructural observations in a fixative cocktail consisting of 1% $OsO_4$ and 2% glutaraldehyde in 0.45 $M$ sodium acetate buffer (pH 6.4) with 10% sucrose (Leys and Reiswig, 1998). For scanning electron microscopy, fixed larvae were dehydrated in a graded ethanol series, critical-point-dried with $CO_2$, and coated with gold in an Edwards S150B sputter coater. Up to five larvae were mounted on each stub with clear nail polish and viewed in a Hitachi S-3500N scanning electron microscope at the University of Victoria.

For transmission electron microscopy, the fixed larvae were dehydrated in a graded ethanol series to 70%, stained with 0.5% uranyl acetate in 70% ethanol *en bloc* overnight, desilicified in 4% hydrofluoric acid in 70% ethanol, and then embedded in Epon (Taab 812). Semithin and thin sections were cut on either a Reichert UM2 or a Leica Ultracut T ultramicrotome. Semithin sections were stained with Richardson's (Richardson *et al.*, 1960), mounted in Histoclad, viewed with a Zeiss Axioskop compound microscope, and photographed with a digital DVC camera using Northern Eclipse software. Thin sections were stained with lead citrate and viewed with a JOEL 1010 transmission electron microscope at the University of Queensland, or with a Hitachi 7000 transmission electron microscope at the University of Victoria.

## Results

*Larval release and swimming behavior*

If sponges were placed in an aquarium without flowing seawater, larvae were released at all times of the day, either within 30 min of collection, or when the brood chambers were cut open with a scalpel. Upon release, the larvae swam out of the oscula and directly upward until they reached the surface of the aquarium. In the presence of light, the larvae generally swam forward continuously, corkscrewing or ro-

**Table 1**

*Ontogenetic change in swimming speed of* Reneira *sp. larvae*

| Age of larva (h post release) | 0 | 2 | 4 | 6 | 12 | 24 |
|---|---|---|---|---|---|---|
| Mean swimming speed (cm/s) | 0.14 | 0.18 | 0.16 | 0.12 | 0.12 | 0.07 |
| Number | 19 | 19 | 19 | 15 | 16 | 11 |
| SD | 0.081 | 0.087 | 0.099 | 0.110 | 0.095 | 0.072 |
| Variance | 0.0066 | 0.0076 | 0.0098 | 0.0122 | 0.0091 | 0.0052 |
| *t* test | Zero-h larvae *vs.* 24-h larvae | | | 2-h larvae *vs.* 24-h larvae | | |
| *P* | 0.0043 | | | 0.0001 | | |

tating clockwise (as observed from the posterior end of the larva), with occasional bursts of acceleration for periods of several seconds. Larvae responded to light in an identical manner in all seasons.

If undisturbed, larvae in the laboratory would swim at the surface of the seawater for the first 2–3 h after release. Thereafter, they tended to remain at the bottom of their dish moving forward slowly or rotating in one spot with the anterior end upward. However, as soon as the dish was disturbed by light or movement, larvae younger than 2 days old would begin swimming vigorously forward, often at the surface of the water. They were energetic swimmers for 12 h, until they began a creeping phase along the substrate prior to settlement and metamorphosis. Whereas undisturbed larvae metamorphosed 12 to 24 h after release, larvae that were disturbed periodically generally did not metamorphose until 48 hours after release or longer. Some disturbed larvae never metamorphosed and eventually died after one week. Of more than 100 larvae observed, three swam in the reverse direction with the long posterior cilia leading.

### *Ontogenetic response of* Reneira *sp. larvae to unidirectional light*

Young larvae (<12 hours old) stimulated by light swam energetically in the mid-water column or on the surface of the test aquarium and stopped when they reached a point at which the light intensity fell, from approximately 10%, to 0.1% of the original intensity (from 73 $\mu M \cdot$ photons $\cdot m^{-2} \cdot s^{-1}$ to 1 $\mu M \cdot$ photons $\cdot m^{-2} \cdot s^{-1}$). Older larvae (>12 h old) swam slowly along the substrate away from the light source and continued swimming until they reached the end of the test aquarium, regardless of light intensity. The mean velocities of newly released larvae (0 h) and of 2-h-old larvae stimulated by light were significantly faster than those of day-old larvae (Table 1).

The great majority of newly released larvae (0 h old) were negatively phototactic in response to unidirectional light [mean angle swum ($a$) = 193 °], but a few larvae in this age cohort swam erratically, showing no preference for swimming direction ($r$ = 0.6) (Fig. 3). Larvae aged 2, 4, and 6 h were all strongly negatively phototactic (Fig. 3). The mean angle swum by larvae in response to light shone from zero degrees was 163 ° ($r$ = 0.9) for 2-h-old larvae, 160 ° ($r$ = 0.82) for 4-h-old larvae, and 174 ° ($r$ = 0.85) for 6-h-old larvae. At 12 h after release, active larvae were still swimming directly away from light [mean angle swum ($a$) = 187 °], while less active larvae swam in spirals in one place and were only weakly phototactic, if at all. By 24 h after release from the brood chambers, the swimming directions of larvae were highly varied ($z$ = 1.637; $r$ = 0.233). All 48-h-old larvae showed little swimming activity and sank to the bottom of the test aquarium rotating gently in one spot (Fig. 3).

### *Response of larval cilia*

Most larval cilia are 20 $\mu$m long and beat in a pattern of metachronal waves that proceeds obliquely around the larva from anterior to posterior pole (Fig. 1d). This beat is unceasing, and the pattern of beat did not change when the larva was prodded or even cut in half. Moreover, these cilia did not respond to changes in light intensity or increased levels of KCl.

The circular band or ring of long posterior cilia circumscribing the unciliated posterior pole beat either intermittently or in a single wave in a counterclockwise direction (as viewed from the posterior of the larva). The beat of these long cilia was unaffected by mechanical stimuli, but when the larva was transected medially, so as to isolate the posterior portion, these cilia stopped beating, apparently because they were tangled in mucus and cellular debris released from the wound. The debris disappeared within a few minutes, and the long posterior cilia resumed their beat. Treatment with seawater containing 10 and 30 m$M$ KCl had no effect on the long cilia, but treatment with seawater containing 50 m$M$ KCl caused the long posterior cilia to stop beating and the larva to stop swimming for several seconds.

The beat of the long posterior cilia halted instantly when the light intensity abruptly increased or decreased. With a sudden increase in light intensity (2.3 to 19.5 $\mu M \cdot$ photons $\cdot m^{-2} \cdot s^{-1}$; 19.5 to 57.7 $\mu M \cdot$ photons $\cdot m^{-2} \cdot s^{-1}$; 57.7 to 100.9 $\mu M \cdot$ photons $\cdot m^{-2} \cdot s^{-1}$; 100.9 to 144.2 $\mu M \cdot$

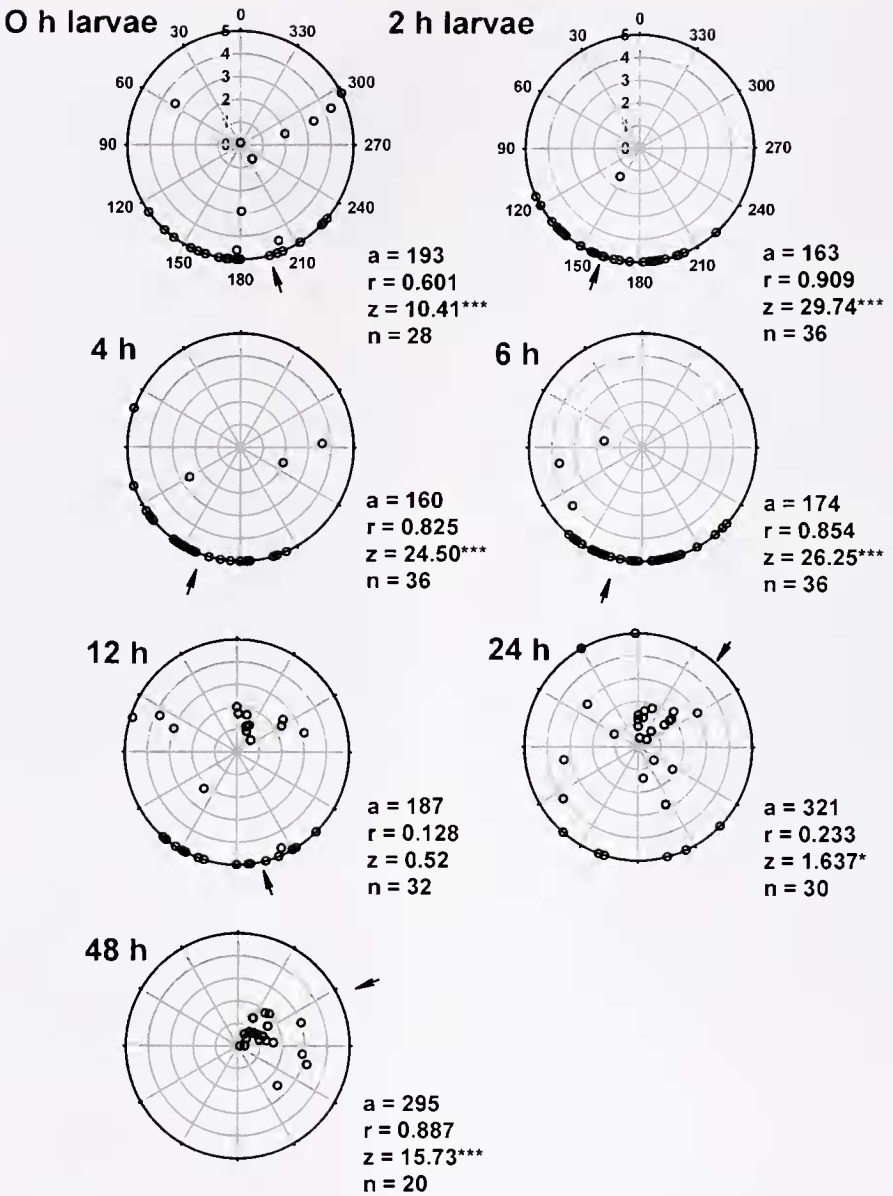


**Figure 3.** Circular histograms showing the directions swum by individual larvae in response to diffuse light shining from zero degrees (see methods for a complete description). The mean angle swum by larvae of an age cohort is given (a) and is shown with an arrow. A Raleigh's test ($z$) determined the degree of dispersion of the data; highly grouped data [a high value of $z$, or a regression ($r$) approaching 1] are significant (***) at $P < 0.001$. The number of larvae ($n$) used at each time point is given. The distance swum by larvae is given in centimeters and displayed as distance from the center of the circle. The great majority of larvae younger than 12 h old swam directly away from the light source, while 12-h-old larvae either swam directly away from the light or were indifferent. The majority of day-old larvae showed no clear phototaxis, while 2-day-old larvae sank to the bottom of the test aquarium and rotated in one spot.

photons $\cdot$ $m^{-2} \cdot s^{-1}$), these cilia immediately straightened and remained straight for several seconds (Fig. 4). If the light intensity remained high for longer than 5 s, the ring of long posterior cilia gradually bent down over the bare posterior pole; the cilia constituting the ring responded sequentially, producing a wavelike motion. The ciliary ring remained bent until the light intensity was gradually reduced, whereupon the cilia began to beat freely again, as though swimming. If the light intensity was suddenly reduced by reversing the gradients described above, the ciliary ring rapidly bent over the bare posterior pole. If the light remained low for more than 5 s, the cilia slowly straightened again in a wavelike motion and remained rigidly extended until the light intensity was gradually increased. The re-

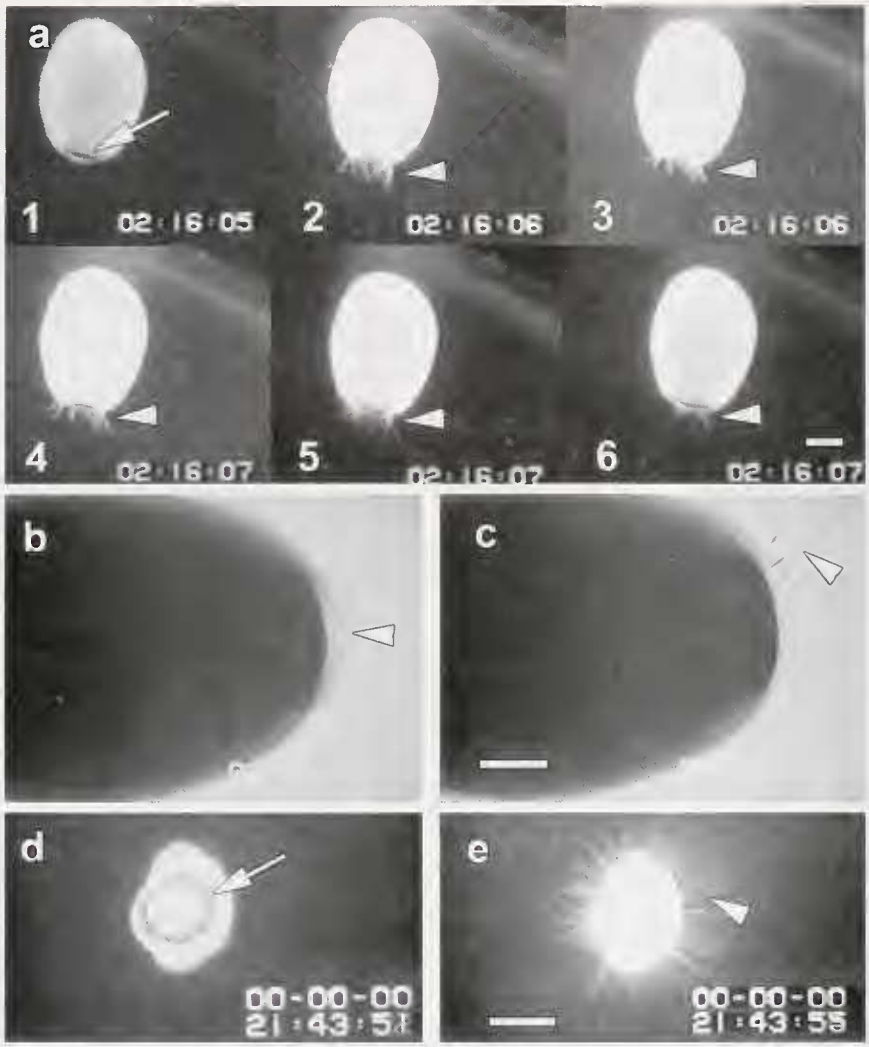


**Figure 4.** The response of the ring of long posterior cilia to a rapid increase or decrease in light intensity (video microscopy). The time that each video frame was captured is shown in the bottom right-hand corner of each image in hours, minutes, and seconds. The rate of straightening and bending of the ring of long posterior cilia shown in all parts of this figure was controlled by the rate that light intensity was increased and decreased. See methods for details. (a) Frame 1: The cilia are bent over the pigment ring (the dark line indicated by the arrow) in response to a previous sudden reduction of light intensity. Frames 2–3: Upon an abrupt increase in light intensity, the long posterior cilia that constitute the ciliary ring (arrowheads) rapidly straighten and remain rigidly extended (frame 3). Frames 4–6: When the light intensity is suddenly reduced, the ciliary ring (arrowheads) rapidly bends down over the pigment ring. (b, c) The ciliary response was viewed with a compound microscope. The long posterior cilia (arrowheads) are bent over the pigment ring when light is abruptly reduced (b), and straighten when the light intensity is rapidly increased (c). (d, e) The long posterior cilia on the posterior portion of a bisected larva still respond to an abrupt increase and decrease in light intensity. (d) The cilia are bent over the pigment ring (arrow) after a previous sudden decrease in light intensity. (e) With an abrupt increase in light intensity the cilia (arrowhead) straighten. Bar: a, d, e: 100 $\mu$m; b, c: 50 $\mu$m.

sponse of the long posterior cilia to changes in light intensity was instantaneous, and the ciliary ring could be made to straighten and bend in unison as fast as a shutter in front of the cold light source could be opened and closed. If the shutter was opened and closed at a slower rate, the cilia straightened and bent more slowly, but still in unison.

The long posterior cilia on isolated posterior portions of the larva, or on posterior portions in which the ciliary ring had been completely bisected, responded in an identical manner. The response of these cilia became increasingly slow in larvae older than 24 h, but even a larva that had settled on its anterior end and was undergoing metamorphosis would continue to move its long posterior cilia in response to changes in light intensity.

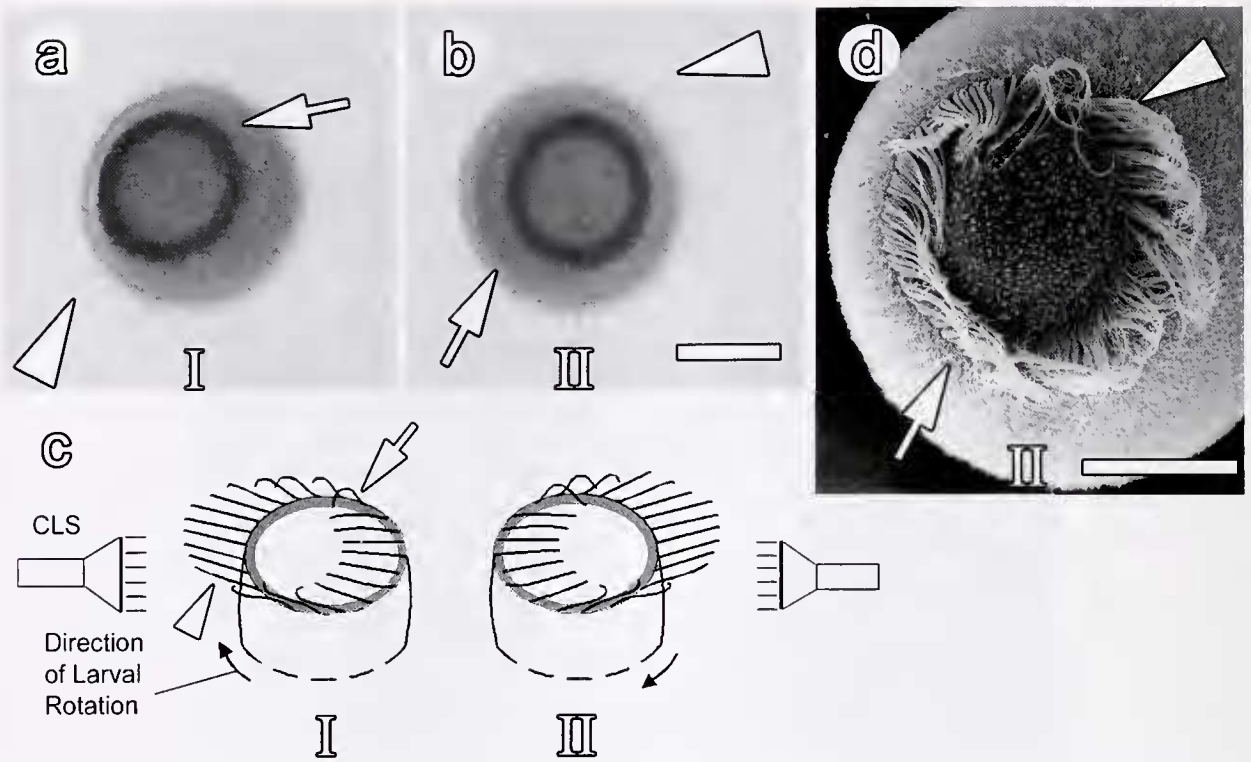


**Figure 5.** Ciliary movement in "half" (bisected) larvae that were rotating white illuminated from the left (I) or right (II) side. (a, b) Frames from a video recording of the posterior portion of a bisected larva rotating in the same spot while illuminated from the left (a) and right (b) as diagrammed in (c). As the larva rotates in a clockwise direction the cilia straighten (arrowheads) when they are closest to the cold light source (CLS), and bend (arrows) over the pigment ring and bare posterior pole when they are farthest from the light source. Magnification of (a) and (b) is the same. (d) A scanning electron micrograph of a larva that was fixed while rotating in illumination from the right shows that the cilia are straight (arrow) on the right and bent (arrowhead) over the pigmented ring on the left. Bar: a, b, d: 100 $\mu$m.

Light shining parallel to the bench top, from either the left or right side of the microscope stage, onto the posterior end of a bisected larva that was rotating in one spot, caused the long posterior cilia closest to the light source to straighten, and those farthest from the light source to bend (Fig. 5). The bisected larvae completed a full rotation once every 1.5–2 s; each long posterior cilium straightened at the instant it reached the side closest to the light source, and bent at the instant it reached the side furthest from the light source. This experiment was readily repeatable with any number of bisected larvae.

*Larval ultrastructure*

Semithin longitudinal sections of the larva revealed three layers (Fig. 6a, b). Uniciliated columnar epithelial cells form the outer layer that constitutes all but the anterior and posterior poles. These cells have two zones: a basal region with a nucleus (2 $\mu$m long) and electron-lucent inclusions (0.66 $\mu$m in diameter), and an apical region that is rich in mitochondria and gives rise to a 20-$\mu$m-long cilium (Fig. 6c). Large mucous cells occur throughout the epithelial layer (Fig. 6c). In the anterior third of the larva, flask-shaped ciliated cells are regularly interspersed among the columnar epithelial cells. These cells have a large, centrally located nucleus, numerous small clear vesicles in the cytoplasm, and possess a cilium that arises from a deep indentation in the apical surface of the cell (Fig. 6d).

Underlying the layer of columnar epithelial cells is a region of cells and collagen that is arranged circumferentially around the larva, perpendicular to the longitudinal axis, giving the impression of a belt or girdle of cells (Fig. 6b). This sheet of cells is interrupted only at the posterior end of the larva. These long, narrow cells contain spherulous inclusions (Fig. 6f). The interior of the larva is composed of at least four cell types, which are aligned along the anterior-posterior axis of the larva and are surrounded by a thick layer of collagen fibers and a single type of rod-shaped bacteria that was present in all specimens sectioned (Fig. 6e, inset). The anterior end of the larva is bare (Fig. 7) and is formed of large, almost cuboidal cells filled with very small (0.08–0.25 $\mu$m), clear vesicles (Fig. 7b, c). Although most of these cells appear to lack cilia, occasional cilia were seen

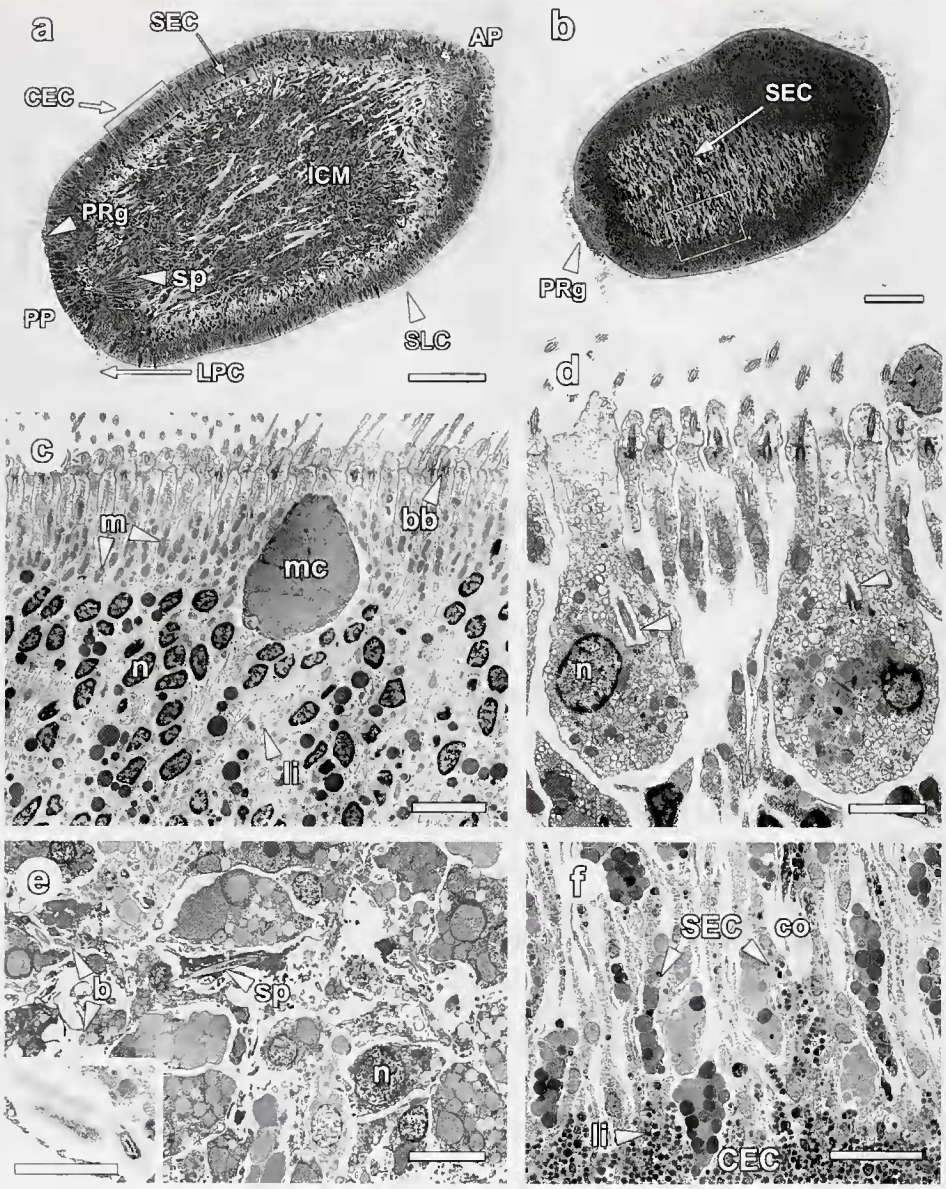


**Figure 6.** The structure and ultrastructure of *Reneira* sp. larvae (a, b: light microscopy; c–f: electron microscopy). (a) A longitudinal section through a 2-h-old larva shows that short (20-$\mu$m-long) lateral cilia (SLC) arise from columnar epithelial cells (CEC) except at the anterior pole (AP) and posterior pole (PP), which are bare. Long posterior cilia (LPC) arise from pigment-filled columnar epithelial cells primarily in the anterior portion of the pigment ring (PRg). Inside the CECs is a layer of subepithelial cells (SEC) that run circumferentially around the larva. Cells in the central region (inner cell mass, ICM) are aligned along the anterior-posterior axis of the larva. Spicules (sp) are evident at the posterior pole. The region in the box is shown in (c). Bar a, b: 100 $\mu$m. (b) A tangential longitudinal section through the edge of a 2-h-old larva shows that the subepithelial cells (SEC) are aligned perpendicular to the A-P axis of the larva. The region in the box is shown in (f). Pigment ring, PRg. (c) Columnar epithelial cells from the region of the larva shown in the box in (a): mitochondria, m; mucous cell, mc; basal body of the cilia, bb; nucleus, n; light inclusions, li. Bar: 4 $\mu$m. (d) Flask-shaped epithelial cells that occur towards the anterior end of the larva possess a large centrally located nucleus (n) and a cilium that arises from a deep invagination in the cell (arrowheads). Bar: 2 $\mu$m. (e) Cells of the inner cell mass. Spicules, sp; nucleus, n; extracellular rod-shaped bacteria, b (inset). Bar: 5 $\mu$m; inset: 2 $\mu$m. (f) Subepithelial cells (SEC) from the region shown in the box in (b) lie in a dense bed of collagen (co). Light inclusions (li) can be seen in the bases of the columnar epithelial cells (CEC). Bar: 10 $\mu$m.

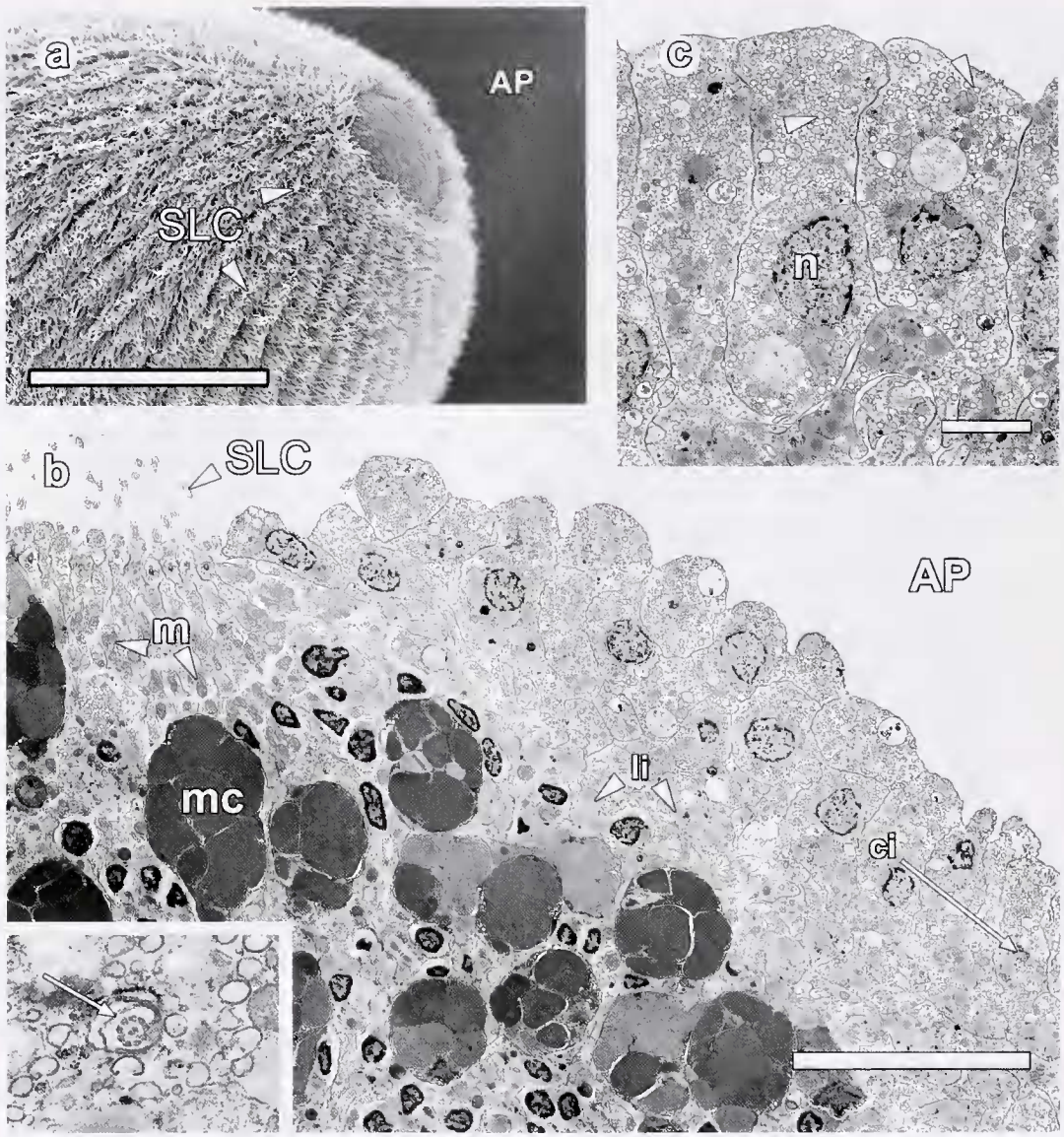


**Figure 7.** Ultrastructure of the anterior pole of *Reneira* sp. larvae. (a) A scanning electron micrograph of a 2-h-old larva shows that the anterior pole (AP) is bare of cilia, and that the short lateral cilia (SLC) are preserved in bands illustrating the metachronal waves entrained by their beating when alive. Bar: 100 μm. (b) A transmission electron micrograph of a region near the edge of the anterior pole of a 48-h-old larva. The short lateral cilia (SLC) mark the end of the columnar epithelial cells at the anterior pole (AP). The anterior-most cells are generally nonciliated, but the occasional cilium (ci, arrow; inset) can be found deep within the cells. Mucous cells, mc; mitochondria, m; light inclusions, li. Bar: 10 μm. (c) Magnification of the cuboidal cells at the anterior end of a newly released (zero hour) larva shows numerous clear vesicles (arrowheads). n, nucleus. Bar: 2 μm.

arising from deep invaginations in the apical surface of the cuboidal cells (Fig. 7b, inset).

At the posterior end of the larva, large cells containing electron-dense, mucus-like inclusions protrude slightly from the bare posterior pole (Fig. 8). At the boundary between these large posterior cells and the columnar epithelial cells with short cilia lie the pigmented cells bearing the long cilia (Fig. 8a). Electron-dense pigment vesicles occur throughout the length of these cells and in the protrusions of their apical surfaces that extend over the base of the neighboring cells, covering the basal portions of the long posterior cilia (Fig. 8, 9a). The posterior-most pigment-filled cells appear to lack cilia, but otherwise most pigmented cells also give rise to a long posterior cilium (Fig. 8, 9a). No obvious changes in the number or size of pigment vesicles, or the area they occupy in the cell protrusions, could be found in thin sections of the posterior of newly released larvae and 2- to 3-day-old larvae. Further, neither

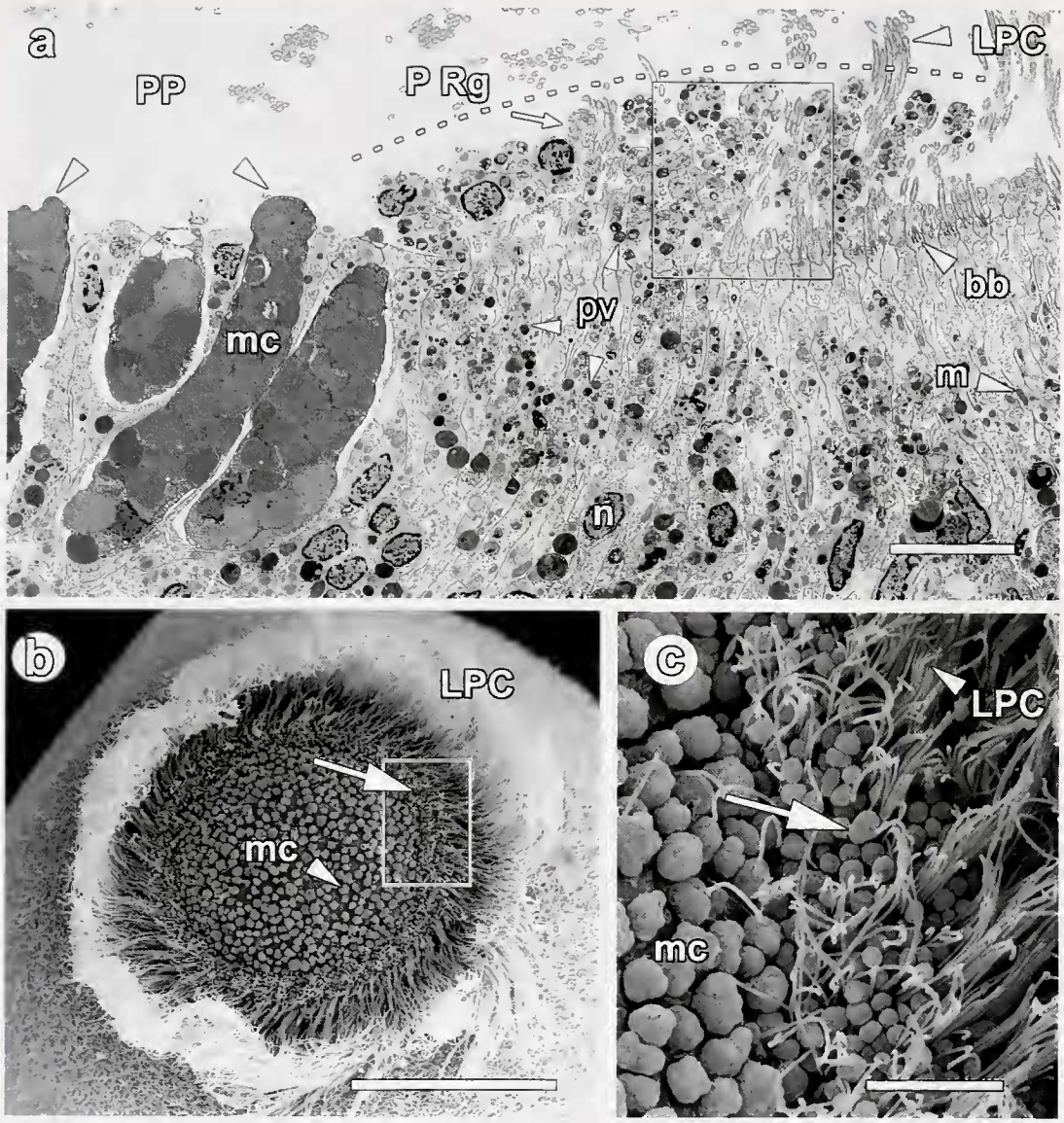


**Figure 8.** Ultrastructure of the posterior pole of *Reneira* sp. larvae. (a) The posterior pole (PP) is formed in part of large mucus-like cells (mc) that protrude slightly from the posterior end (arrowheads). The pigment ring (PRg, dashed line) is formed of columnar epithelial cells with protrusions (arrow) at their apical surface. These cells contain numerous pigment vesicles (pv) throughout their length and in the apical protrusions. The long posterior cilia (LPC) arise primarily from the anterior-most of these cells. Magnification of the region in the box is shown in Figure 9a. Mitochondria, m; basal bodies of the cilia, bb; nucleus, n. Bar: 5 $\mu$m. (b) A scanning electron micrograph of the posterior pole of a larva that has been fixed while exposed to an abrupt increase in light intensity to cause the long posterior cilia to straighten. Note also that the mucus-like cells (mc) protrude slightly from the posterior end (arrowhead), and that most long posterior cilia (LPC) are anterior to the pigment-filled protrusions (arrow). Magnification of the region in the box is shown in (c). Bar: 100 $\mu$m. (c) A scanning electron micrograph of the region of the pigment ring shown in the box in (b). Mucus-like cells (mc) protrude from the posterior pole, and pigment-filled protrusions (arrow) lie at the base of, and slightly posterior to, the long posterior cilia (LPC). Bar: 20 $\mu$m.

the number of pigmented cells nor the general histology of the posterior end in older larvae changed. The structure of the basal bodies and of the basal portions of the long posterior cilia did not appear to be different from those of the short lateral cilia (Fig. 9b, c).

## Discussion

This report presents the first demonstration that sudden changes in light intensity cause an instantaneous response in the cilia of a sponge larva. This, together with the demon-

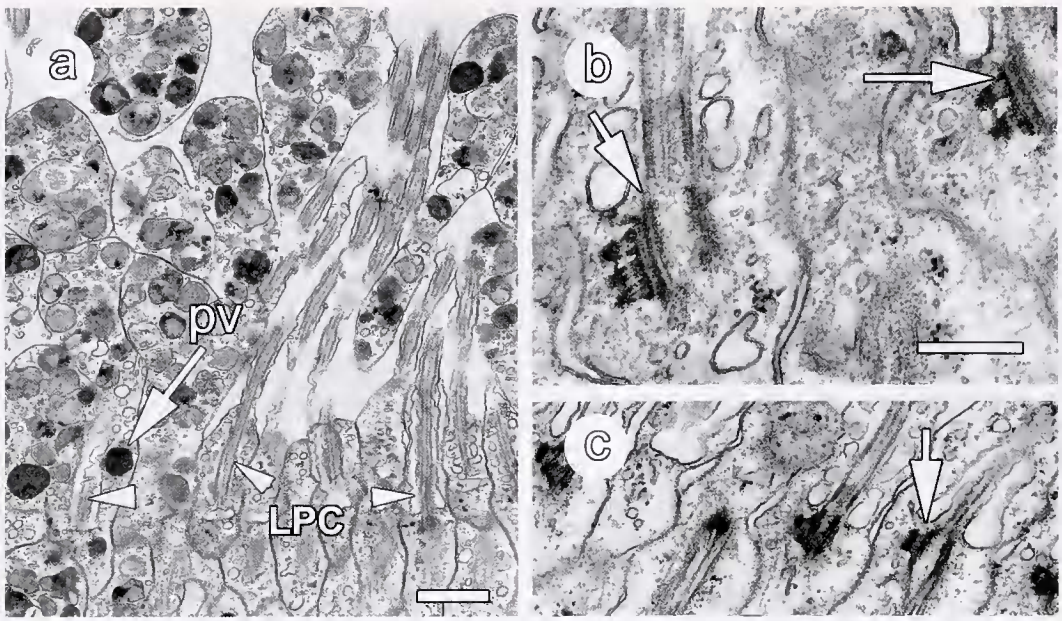


**Figure 9.** Details of the pigmented cells and ciliated cells in and near the pigment ring (transmission electron microscopy). (a) Magnification of the boxed region in Figure 8a showing that at least some pigment-filled vesicles (pv; arrow) are in protrusions of the same cells that give rise to the long posterior cilia (LPC, arrowheads). Protrusions of the apical surface of other cells in the ciliated ring are also in view in this section. Bar: 1 μm. (b) Basal bodies (arrows) of the long posterior cilia. (c) Basal bodies (arrow) of the short lateral cilia. Bar b, c: 0.5 μm.

stration that light shining at an oblique angle on the long posterior cilia of a rotating larva causes the cilia nearest the light to straighten and those furthest from the light to bend as the larva rotates, implicates the posterior pigment ring and the band of long cilia in steering the sponge larva away from bright light.

*Sponge larval "behavior"*

Given that cellular sponges lack neurons and gap junctions (Pavans de Ceccatty, 1974a; Mackie, 1979; Lethias *et al.*, 1983; Green and Bergquist, 1982; Woollacott, 1993), sponge larval behavior is usually explained as being due to the physical attributes of the larva. For example, many sponge larvae are reported to swim directly upward after release from the adult (Bergquist and Sinclair, 1968; Wapstra and van Soest, 1987), although there is no evidence that sponge larvae possess gravity or pressure sensors, such as statocysts, or a conduction system that would allow them to translate such messages rapidly into behavior. However, Warburten (1966) suggested that the ability of young larvae to swim to the top of a tube of seawater each time it was inverted, whether illuminated from above or below, could be caused by a differential weighting of the larva at the posterior end. Indeed, as in many species, spicules develop at the posterior end of *Reneira* sp. larvae after their release from the adult, and Maldonado *et al.* (1997) provided experimental evidence that differential weighting, caused by the presence of spicules at the posterior end in some larvae, is correlated with positive geotaxis and rheotaxis.

The beating of cilia in metachronal waves that run obliquely around the long axis of the larva is often thought to be a result of coordinated behavior (Borojevic, 1969). However, the entrainment of cilia into metachronal waves in many animal systems has been demonstrated to be caused by viscous coupling among cilia (Sleigh, 1974). A very small number of *Reneira* sp. larvae do swim backwards, suggesting that reversal of the direction of metachronal waves is possible in *Reneira*.

*Phototaxis and the shadow response*

Neither of the above examples of sponge larval behavior suggests that sensory receptors are involved. For this reason, the role of the long posterior cilia in *Reneira* sp. larvae in responding to changes in light intensity, and thus in steering the larva away from the light, is intriguing. Although photoreceptors have often been implicated in the phototaxis of sponge larvae (Kaye and Reiswig, 1991; Woollacott, 1990, 1993; Maldonado and Young, 1996, 1999), the mechanism by which this might occur has not been explored by any of these authors.

The different responses of old and young larvae when swimming into a shaded region of a test chamber has been

noted previously. Maldonado *et al.* (1997) suggested that older larvae are more sensitive to light than newly released larvae, because they continue to swim long after they have moved into a shaded region. *Reneira* sp. larvae exhibited a similar behavior. However, qualitative analysis of the ultrastructure of the posterior end of larvae of all ages revealed no changes in the number of pigment vesicles, the area of the cells occupied by pigment vesicles, or the number of long posterior cilia. Furthermore, the long posterior cilia responded to changes in light intensity in larvae of all ages, including those undergoing metamorphosis, although the response became more sluggish in older larvae. Another interpretation is that, upon entering a shaded area, the younger larvae exhibit a "shadow response"—a photokinetic response that changes the level of activity rather than the direction of movement. The function of the shadow response has been examined in some detail in ascidian tadpole larvae (Woodbridge, 1924; Grave, 1944; Young and Chia, 1985; Svane and Young, 1989) where it appears to influence the settlement patterns of larvae, and in the hydrozoan medusa *Polyorchis penicillatus* where it is involved in vertical diurnal migration (Spencer and Arkett, 1984; Arkett, 1985). The immediate response of the long posterior cilia of *Reneira* sp. larvae to a sudden decrease in light—bending to cover the pigmented ring and posterior pole—is also suggestive of a shadow response. If larvae exhibited this response when entering a region of greatly diminished light (such as under a rock on the reef flat), the larva would stop swimming forward. This suggests that, contrary to the conclusion drawn by Maldonado *et al.* (1997), older larvae are, in fact, less sensitive than younger larvae to changes in light intensity.

*The light receptor*

Ciliary or rhabdomeric photoreceptors have been described in all invertebrate phyla except Porifera (Eakin, 1968, 1972; Burr, 1984). Both Tuzet (1973) and Amano and Hori (1992) have suggested that the cruciform cells in developing amphiblastulae, the larvae of calcareous sponges, are photoreceptive, but no studies have confirmed this function in larval behavior. The morphology of photoreceptors in basal metazoan groups is unstudied recently, but the work of Eakin (1968, 1972) suggests that the simplest photoreceptors, known from the Cnidaria, are monociliated cells surrounded by cells containing pigment vesicles. The pigment cells in *Reneira* sp. that give rise to the long posterior cilia are similar in structure to the simple photoreceptors described in the hydromedusan *Leuckartiara octona* (Singla, 1974) and to sensory cells that may be photoreceptors in the planulae of *Hydractinia echinata* (Thomas *et al.*, 1987; Weis *et al.*, 1985) and *Phialidium (Clytia) gregarium* (Thomas *et al.*, 1987).

The surface of the pigment cells protrudes out over the surrounding epithelium forming a dark ring on the posterior side of the long cilia. This band of pigment would effectively block light coming from across the bare posterior pole from reaching the basal portion of the cilium (Figs. 8, 9). It appears that although the posterior-most pigment-containing cells may lack cilia, most cells possess both pigment and a long cilium. Although the location of the photoreceptor is currently unknown (future work using microspectrophotometry to determine its location being planned), the cilium is probably both the receptor and effector, as in the well-studied green unicell *Euglena* (Eakin, 1972; Naitoh and Eckert, 1974; Neuman and Hertel, 1994). The effect of increased external potassium ion concentration in causing temporary arrest of the long cilia in *Reneira* sp. larvae suggests that depolarization of the membrane potential, and possible influx of calcium into the cilium, is the mechanism behind the shadow response of sponge larvae. The phenomenon of reversal or inhibition of ciliary beating due to calcium ion influx resulting from membrane depolarization, is well known in protists (reviewed by Naitoh and Eckert, 1974; Eckert *et al.*, 1976), ctenophores, anthozoans, bivalve gills, echinoderm pleutei, and pelagic tunicates (reviewed in Aiello, 1974).

As indicated earlier, unlike planulae, parenchymellae lack neurons or gap junctions that would allow coordination of signals between the cells with long cilia. Instead, each posterior ciliated cell probably responds independently to changes in light intensity. On the basis of the overt responses of the long posterior cilia to abrupt changes in light intensity, and the asymmetric response of the long posterior cilia to light shining on the cilia from the side, as shown in Figure 5, we hypothesize that the larva steers by the subtle photokinetic responses of each ciliated cell to the light, as diagrammed in Figure 10. As the larva rotates through the water, the base of those cilia on the side opposite the direction of the light would be shadowed by pigment, thus triggering a shadow response, which would cause those cilia to bend and cover the pigmented ring (Fig. 10 B arrowhead). Again, as the larva rotates, cilia whose bases are exposed to light would straighten and beat (Fig. 10 B arrow), thus steering the larva away from the light. In this manner, no coordination between cells is required to steer the larva. Rather, a cumulative effect is achieved by the slightly different angle at which each cilium is exposed to or shaded from light. Phototaxis in *Euglena* is thought to be based similarly on the shading of its photoreceptor (Doughty, 1993). However, as steering in *Euglena* has also been shown to depend largely upon polarized light (Creutz and Diehn, 1976; Häder, 1993), such mechanisms of receiving light cues should also be considered in further investigations of the photoreceptor in sponge larvae.

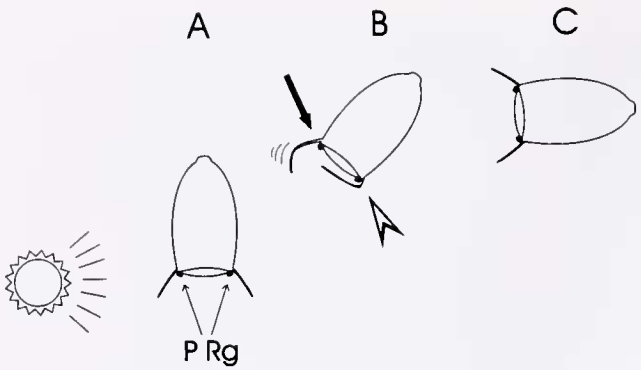


**Figure 10.** Diagram describing the suggested mechanism by which the pigment ring and long posterior cilia allow *Reneira* sp. larvae to steer away from a light source. (A) As the larva rotates, light from one side of the larva impinges on the base of the cilia closest to the light, but is blocked by the pigment ring from the cilia furthest away from the light. (B) Cilia exposed to the light (arrow) straighten or beat rapidly, depending on the extent of their exposure; those hidden from the light by pigment (arrowhead) undergo a shadow response and bend over the pigment ring. (C) The individual response of each cilium to light as the larva rotates causes a graded response taking the larva away from the source of light.

*Coordination of behavior and cellular differentiation in sponge larvae*

Cellular differentiation is integral to the behavior of *Reneira* sp. larvae. Five regions of the larva are distinctly differentiated (Fig. 11). The outer ciliated columnar epithelial layer of the larva is separated from the cells in the central region by a sheath or band of circumferential cells. A radial or circumferential sheath has been described in many parenchymella larvae as a subepithelial cell layer (Meewis, 1941; Brien, 1973; Woollacott, 1993). Although it has been suggested that the cells in this layer have a secretory function (Meewis, 1941), it is equally possible that, in light of the paucity of cell-cell junctions in these larvae, the circumferential subepithelial cells give structural support to the larva during release from the parent and during swimming. The cells of the anterior pole are differentiated in *Reneira* sp. larvae as well. Both the monociliated ciliated flask-shaped cells that occur towards the anterior end of the larva and the large cuboidal cells at the anterior pole have numerous small clear vesicles and may therefore have a secretory function. However, the presence of a cilium arising from a deep invagination in both cell types is also reminiscent of some sensory cells in gastropod larvae (*e.g.*, Kempf *et al.*, 1997). This anterior region attaches to the substratum at settlement in *Reneira* sp. larvae. Finally, although the function of the large cells at the posterior pole remains unclear, the pigmented epithelial cells from which arise the long posterior cilia are clearly differentiated to steer the larva away from light.

The resulting picture of the sponge larva is not one typically conjured up of a parazoan, an "almost metazoan." The *Reneira* sp. larva is an ensemble of differentiated and pluripotential cells arranged in stereotypic patterns along both central-lateral and anterior-posterior axes (Fig. 11). The spatial arrangement of differentiated cell types in the larva, with their specific functions and behaviors, plays a central role in guiding the larva to a suitable settlement location. Clearly this grade of multicellular organization is built by the functioning of multiple transcriptional networks during embryogenesis and larval development. Although a variety of regulatory genes are known to exist in sponge genomes (*e.g.*, Degnan *et al.*, 1993, 1995; Seimiya *et al.*, 1994; Coutinho *et al.*, 1994; Kruse *et al.*, 1994; Hoshiyama *et al.*, 1998), and even though they may be locally expressed in the larva, it is unclear whether conserved genes involved in bilaterian development are operating in a similar manner in sponges. Analysis of the sponge larva and its embryogenesis may enable the identification of developmental genes and processes that are shared among all metazoans, helping us to understand the earliest steps in animal evolution.

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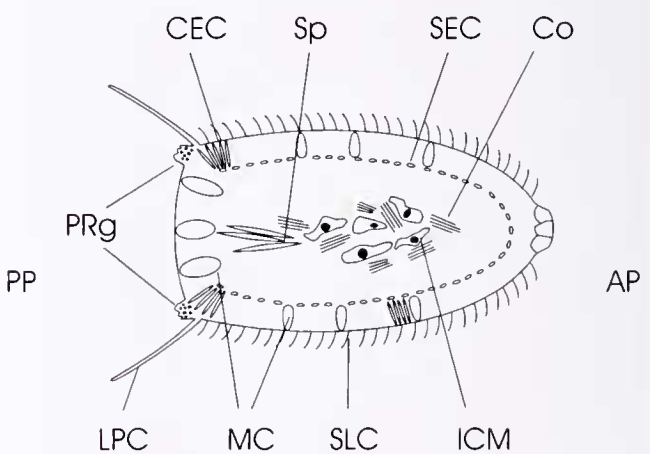


**Figure 11.** Schematic diagram of cellular differentiation in *Reneira* larvae. PP, posterior pole; AP anterior pole; MC, mucous cell; PRg, pigment ring; LPC, long posterior cilia; SLC, short lateral cilia; CEC, columnar epithelial cells; SEC, subepithelial cells; ICM, inner cell mass; Co, collagen; Sp, spicules.

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